FEBS Letters 583 (2009) 1649–1652

FEBS Letters
journal homepage: www.FEBSLetters.org

The impact of PEPC phosphorylation on growth and development of *Arabidopsis thaliana*: Molecular and physiological characterization of PEPC kinase mutants

Patrice Meimoun [a,1], Aurélie Gousset-Dupont [a,1], Bénédicte Lebouteiller [a], Françoise Ambard-Bretteville [a], Evelyne Besin [a], Caroline Lelarge [b], Caroline Mauve [b], Michael Hodges [a], Jean Vidal [a,*]

[a] *Institut de Biotechnologie des Plantes, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8618, Bâtiment 630, Université Paris-Sud 11, 91405 Orsay Cedex, France*
[b] *Plate-forme Métabolisme-Métabolome, Institut Fédératif de Recherche 87, Bâtiment 630, Université Paris-Sud 11, 91405 Orsay Cedex, France*

ARTICLE INFO

Article history:
Received 28 January 2009
Revised 9 April 2009
Accepted 20 April 2009
Available online 4 May 2009

Edited by Michael R. Sussman

Keywords:
Metabolomic
Phosphoenolpyruvate carboxylase
Phosphoenolpyruvate carboxylase kinase
mutant
Arabidopsis thaliana

ABSTRACT

Two phosphoenolpyruvate carboxylase (PEPC) kinase genes (*PPCk1* and *PPCk2*) are present in the *Arabidopsis* genome; only *PPCk1* is expressed in rosette leaves. Homozygous lines of two independent *PPCk1* T-DNA-insertional mutants showed very little (dln1), or no (csi8) light-induced PEPC phosphorylation and a clear retard in growth under our greenhouse conditions. A mass-spectrometry-based analysis revealed significant changes in metabolite profiles. However, the anaplerotic pathway initiated by PEPC was only moderately altered. These data establish the *PPCk1* gene product as responsible for leaf PEPC phosphorylation *in planta* and show that the absence of PEPC phosphorylation has pleiotropic consequences on plant metabolism.

1. Introduction

Various protein kinases have been shown to catalyse the N-terminal phosphorylation of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) *in vitro*: a calcium-dependent protein kinase [1,2], the catalytic subunit of the mammalian protein kinase A [3] and a calcium-independent protein kinase named PEPCk [1,4], which was considered to be the PEPC kinase acting *in planta*. The PEPCk is a small (32 kDa), very low-abundance protein controlled at the turnover level in leaves of C_4, crassulacean acid metabolism (CAM) and C_3 plants [4]. Recent results have shown that it is encoded by a small gene family with variable expression in relation to plant organ and environmental factors [4–10]. Two genes, At1g08650 (*PPCk1*) and At3g04530 (*PPCk2*), with a single intron located close to the 3′ end, have been identified in the *Arabidopsis* genome. RT-PCR experiments have shown that *PPCk1* is mainly expressed in leaves, while *PPCk2* is more expressed in roots and flowers [8].

The N-terminal phosphorylation of PEPC is believed to play a crucial role in regulating the enzyme activity (higher activity and lower sensitivity to L-malate at the physiological pH) and, thus, carbon assimilation (C_4 and CAM) and carbon flux through the anaplerotic pathway. However, CO_2 assimilation rates were not affected in transgenic lines of the C_4 species *Flaveria bidentis* deprived of the PEPCk and containing low levels of phosphorylated C_4-PEPC [11]. This surprising result raised the question of the physiological role of the modulation process in plants.

The goal of the present work was to clarify the role of PEPC phosphorylation in the context of the anaplerotic pathway in rosette leaves of the C_3 plant *Arabidopsis* [12]. This has been assessed following the isolation and the molecular characterization of two independent homozygous *PPCk1* insertional mutant lines.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (ecotype Wassilevskija) was grown from seed in a greenhouse (8 h photoperiod or 16 h photoperiod, 20 °C day/18 °C night) under 50 μE m^{-2} s^{-1} at the leaf level and 60% relative humidity, during the winter months. Plants were watered daily with standard nutrient solution: Hydrokani C2 (5 ml/L of water: Hydro Agri France).

Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEPCk, PEPC kinase; CAM, crassulacean acid metabolism; WT, wild-type; P-PEPC, phosphorylated-PEPC; OAA, oxaloacetate

* Corresponding author. Fax: +33 1 69153423.
E-mail address: jean.vidal@u-psud.fr (J. Vidal).
[1] Authors having equally contributed to this work.

0014-5793/$36.00
doi:10.1016/j.febslet.2009.04.030

2.2. Isolation and genotyping of pepck1 mutants

Homozygous *ppck1* mutant alleles (*dln1* and *csi8*) were isolated from the Versailles T-DNA mutant library (INRA Versailles, France) by the successive PCR screening of available genomic DNA pools using the following primers: PK5, 5′-AAAGGTAGACCGACTCAAGAG-3′; PK3, 5′-CTCACATCCTTTTACTCACCCC-3′; Tag5, 5′-CTACAAATTGCCTTTTCTTATCGAC-3′. For *dln1*, segregation studies of heterozygous plants based on kanamycin resistance showed a Mendelian inheritance of the character and Southern experiments detected the presence of a single Bar gene (data not shown). This strongly indicates that the *dln1* mutant contains a single insertion in the genome. Southern experiments also showed the occurrence of two T-DNA insertions in the *csi8* DNA (data not shown). Since both phenotypes (*dln1* and *csi8*; see below) are very similar, this suggests that the second insertion in *csi8* genome is mostly neutral with respect to the physiological context studied in this work. Thus, the observed phenotype in both *dln1* and *csi8* can be attributable to the PEPCk1 mutation.

2.3. RNA extraction and RT-PCR experiments

Total RNA was extracted from 100 mg of frozen, powdered leaves by the TRIzol method (Invitrogen). Reverse transcription was performed with 1 μg total RNA, 200U MLV reverse transcriptase RNase H minus (Promega) and associated buffer, 0.5 mM dNTP, and either 10 μM 3′ primer (PK31: 5′-TCGCCAAACTCAGAAGCAG-3′) or 10 μM oligodT primer, 1 h at 42 °C. Subsequent PCR experiments were performed using a thermocycler (GeneAmp® PCR system 9700, Applied biosystem) in a 20 μL final volume containing 1/10th of the reverse transcription product, 0.05U Taq polymerase (Qiagen) and associated buffer, Q buffer 1X, 1 mM $MgCl_2$, 0.5 mM dNTP and 10 μM of the following primers: PK31 and PK135 (5′-AGCCGAACAAGCATTAGGG-3′), to amplify the 3′ region of *PPCk1*; PK31 and PK15 (5′-TGATATTATGACTTGCAGCC-3′), to amplify the coding region of *PPCk1*; PK15 and PK3i (5′-CTTTAGCCATAGATGAAACCC-3′) to amplify the coding region up to the intron; PK25 (5′-CTGCAGAGGATGCTCTTC-3′) and PK23 (5′-ATTGCTGAAACACACAAC-3′) to amplify *PPCk2*. To amplify actin transcripts the following primers were used: Act2up (5′-TTCCCTCAGCACATTCCAGCAG-3′) and Act2do (5′-TTAACATTGCAAAGAGTTTCAAGG-3′). After 5 min at 94 °C, 35 PCR cycles were carried out: 45 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C, then 7 min at 72 °C.

2.4. Protein extraction and PEPC assays

Non-denaturing and denaturing protein extraction, determination of protein amounts, enzyme assays and malate effect were as described in [13].

2.5. Phospho-protein purification and PEPC detection by Western blotting

Phospho-proteins were purified by affinity chromatography using the "Phospho-Protein Purification Kit" (Qiagen) according to [13]. Purified phospho-proteins were analyzed by SDS–PAGE [14] and gel blotting. The PEPC was detected by the N-terminal antibody (1/50 000 dilution) [15] and a peroxidase-based assay "Supersignal West Dura Signal" from Pierce.

2.6. Mass spectrometry (GC-TOF-MS) analysis

The extraction, metabolite derivatization, GC-TOF-MS, data processing and analysis of the metabolite profiles were carried out as described in [16]. Integrated peak areas were obtained after deconvolution by the LECO PEGASUS III software. These were then normalized to the internal standard (ribitol) peak area for each injection. To identify metabolites that showed significant variation, the centre-reduced values were subjected to ANOVA (P 0.05). Of the 78 metabolites identified in *Arabidopsis* leaves, 20 showed significant inter-group variation.

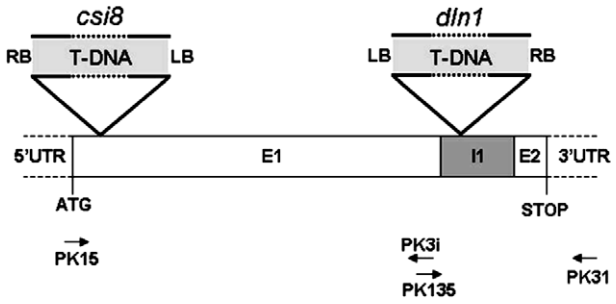

Fig. 1. Position of the T-DNA in the *PPCk1* gene and the primers used for RT-PCR analyses. In *dln1* the T-DNA is inserted in the unique intron (30 bp after the beginning of the intron) of the gene. In *csi8*, the T-DNA is inserted 66 bp after the start codon. The arrows indicate the position of the primers used to study *PPCK1* expression in WT and mutant extracts by RT-PCR. E1, exon1; E2, exon2; I1, intron1; RB, right border; LB, left border; UTR, untranslated region.

3. Results and discussion

3.1. Characterization of leaf ppck1 insertional mutants

Homozygous lines of two independent mutants (*dln1* and *csi8*) were shown to have the T-DNA inserted in the intron (*dln1*: 30 bp after the beginning of the intron) and the first exon (*csi8*: 66 bp after the translation start site) of the *PPCk1* gene (Fig. 1). *PPCk1* transcripts were induced in leaves after an exposure to light, with mRNA detected by RT-PCR after 30 min, after which the transcript levels remained relatively constant during the light period (Fig. 2). Similar RT-PCR experiments carried out using RNA extracted from illuminated plants (180 min after the start of the light period) showed the absence of "full-length" *PPCk1* transcripts in the rosette leaves of *dln1* plants (Fig. 3A, lane 4) compared to the wild-type (WT) that exhibited *PPCk1* mRNA (Fig. 3A, lane 2). Both the mutant and WT leaves did not contain any detectable *PPCk2* transcripts (not shown). However, when using appropriate DNA primers (see Fig. 1), a truncated *PPCk1* transcript presumably lacking the small 48 bp coding sequence of the second exon was detected (Fig. 3A, lane 3, and lane 1 as a control). For the *csi8* mutant having a T-DNA inserted very close to the start codon of the gene, no 'full length' *PPCk1* transcripts were detected (Fig. 3B, lane 2) when compared to the WT (Fig. 3B, lane 1).

3.2. In vivo PEPC phosphorylation

The malate test (based on the altered inhibition of PEPC activity by malate after phosphorylation) strongly indicated the absence of light-induced PEPC phosphorylation in both *ppck1* mu-

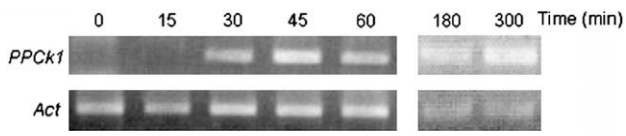

Fig. 2. Time course of *PPCk1* transcript accumulation in leaves of *Arabidopsis*. Dark-treated plants were illuminated (450 μE m^{-2} s^{-1}; time 0) and RNA prepared from leaves collected at the indicated time points. RT-PCR was performed by using primers PK135 and PK31. *Act*: Actin II transcripts (primers: Act2up and Act2do) were used as a control.

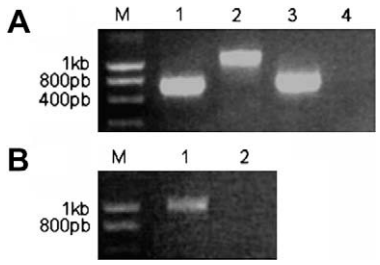

Fig. 3. RT-PCR analysis of *PPCk1* transcripts from wild-type and *ppck1* rosette leaves. (A) Wild type (lanes 1 and 2) and *dln1* (lanes 3 and 4). RNA was extracted from illuminated leaves and RT-PCR was performed using either PK15-PK3i (lanes 1 and 3) or PK15-PK31 (lanes 2 and 4) primers, as described in the Experimental procedures. (B) Wild type (lane 1) and *csi8* (lane 2). RT-PCR was performed using PK15-PK31 primers. M: Molecular mass markers.

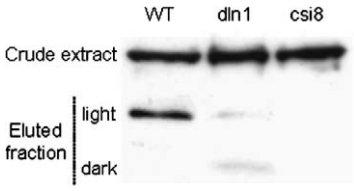

Fig. 4. Affinity chromatography of the phosphorylated PEPC from rosette leaves of *Arabidopsis* wild-type and *ppck1* mutants. Protein extracts were prepared from illuminated or darkened leaves by the denaturing protocol and subjected to phospho-protein affinity chromatography (1.5 mg of total proteins) as described in the experimental procedures. Aliquots of crude extracts (50 μg of proteins) and eluted fractions (10 μg of proteins) were submitted to SDS-PAGE. After transfer to a PVDF membrane, the PEPC protein in crude extracts and the P-PEPC in eluted fractions were detected using a PEPC N-terminal specific antibody and revealed by ECL.

tants (IC_{50} malate: WT: 0.2 mM; *dln1*: 0.08 mM; *csi8*: 0.08 mM). In a previous work, phospho-protein affinity chromatography (using Qiagen affinity columns) combined with gel blot and mass spectrometry analyses showed that phosphorylated PEPC (P-PEPC) could be efficiently separated from non-P-PEPC forms [13]. When denatured protein extracts were applied to the column high amounts of P-PEPC were found in the case of illuminated WT leaves, as expected; it was very low (*dln1*), or undetectable (*csi8*) in the case of the mutants, though the amount of total PEPC protein loaded on the column was similar (Fig. 4). The very low quantity of P-PEPC detected in *dln1* could be due to the fact that a truncated PEPCk1 retains some activity. These findings definitely establish that PEPCk1 is the unique protein kinase that controls the light-dependent increase in PEPC phosphorylation in *Arabidopsis* rosette leaves.

3.3. Effect of the mutation on plant development and metabolite levels

Under our greenhouse conditions during the winter months, both *ppck1* mutants exhibited a slower growth as seen by rosette diameter (Fig. 5) and a retard in flowering time as shown in the delay of floral stem growth when the plants were placed under long-day conditions (Fig. 6). A mass-spectrometer-based metabolite profile analysis was subsequently performed on rosette leaves from five plants harvested (quickly frozen) in the middle of the light period. This revealed multiple impacts of the mutation on several pathways of primary and secondary metabolism. PCA and ANOVA analyses showed that 20 out of 78 metabolite levels showed statistically significant alterations (see selected metabolites in Table 1). In primary metabolism, they mainly concerned (Table 1): the photorespiratory pathway with decreased content of glycolate and glycine, and an increase in serine which leads the gly/ser ratio to decrease. Such change in this ratio may indicate that the pathway flux is increased in mutant leaves [16,17]. This hypothesis is supported by a decrease in glucose and sucrose that reflects a limitation in carbon flux through the Rubisco and the photosynthetic pathway. Also, one clear result is the strong decrease in the Krebs cycle intermediates, succinate and fumarate

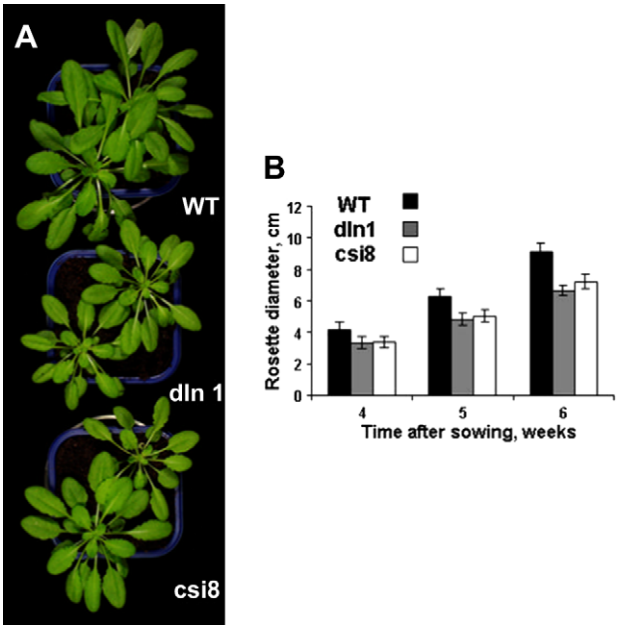

Fig. 5. Developmental phenotype of *ppck1* plants. The photographs shows the difference in rosette size of WT and mutant plants (A) and the quantification by the measurement of rosette diameter (B) (means of 40 plants at three time points after sowing).

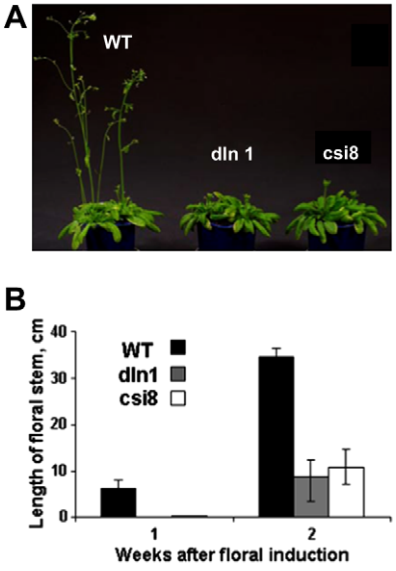

Fig. 6. Flowering time phenotype of *ppck1* plants. After 6 weeks of culture, plants were transferred to long-day conditions to induce flowering. Floral stem development (A) and its quantification (means of 40 plants) at two time points after floral induction (B).

Table 1
Comparative analysis of metabolite levels in wild-type and *ppck1* mutant rosette leaves. Values are the mean of six independent measurements (six different individuals) ± S.E. Metabolites that are significantly different (MEV-ANOVA) to the wild-type (WT) are indicated in bold. The list of metabolites is limited to those that are discussed in the text. All values are relative to the added standard (see Section 2).

Metabolite	WT	Se	dlnl	Se	csi8	Se
PEPC-related, anaplerotic pathway and Krebs cycle						
Aspartate	3.560	0.400	4.040	0.690	3.490	0.740
L-malate	1.810	0.290	1.430	0.220	1.110	0.240
Citric acid	1.260	0.230	1.390	0.250	1.000	0.240
Glutamine	0.410	0.086	0.330	0.049	0.380	0.050
Glutamic acid	1.590	0.160	2.040	0.300	1.680	0.190
Succinic acid	0.840	0.080	0.430	0.040	0.480	0.030
Fumaric acid	62.200	7.990	36.200	4.720	34.300	5.780
Photorespiratory pathway						
Glycolic acid	0.123	0.008	0.085	0.009	0.088	0.004
Glycine	9.690	1.140	3.610	0.370	7.340	0.690
Serine	4.560	0.360	8.190	1.140	5.770	0.170
Glyceric acid	0.187	0.020	0.159	0.014	0.216	0.009
Sugar pathway						
Sucrose	17.700	2.280	13.270	0.980	13.070	1.010
Glucose	8.940	0.840	5.680	0.980	5.920	0.680
Fructose	7.750	0.890	6.050	1.480	6.090	0.750
Secondary metabolism						
Quinic acid	0.050	0.010	0.028	0.004	0.016	0.001
P-hydroxybenzoic acid	0.230	0.027	0.123	0.017	0.105	0.003
Caproic acid	0.302	0.057	0.153	0.023	0.140	0.007

(statistically significant), while malate showed a small but non-significant decrease, and citrate was comparable to the WT. Thus, surprisingly, the upstream part of the cycle leading to 2-oxoglutarate formation (the carbon skeletons required for amino acid synthesis in the chloroplasts) remained unchanged, while metabolite content in the downstream part of the cycle was reduced. The fact that glutamate and glutamine were also found to be unchanged in the *ppck1* mutants indicated that the anaplerotic pathway was not affected. Altogether the results indicate that the anaplerotic pathway is maintained at the expense of the Krebs cycle activity. That the PEPC activity was little affected by the mutation was also suggested by unchanged levels of aspartate (stable), which derives from oxaloacetate (OAA) (unstable and not detected in the metabolite profiles obtained by MS) by a transamination reaction. Such perturbations of the Krebs cycle, the photorespiratory pathway and sugars content could reflect a depressed carbon economy and cellular energy status that may account for the retarded growth and flowering time of the mutants (Figs. 5 and 6). Thus, the results lead to the astonishing conclusion that in *Arabidopsis* the inhibition of PEPC phosphorylation has little or no effect on the metabolic flux through the anaplerotic pathway. Similarly, in transgenic C_4 plant *Flaveria bidentis* where antisense/RNAi technology was used with success to inhibit PEPCk activity and PEPC phosphorylation, no alteration of the functioning of C_4 photosynthesis was observed [11]. In *Arabidopsis*, however, several pathways are clearly perturbed and it can be hypothesized that leaf PEPCk1 has several targets in addition to PEPC. Further work is needed to clarify the physiological role of PEPC phosphorylation and the specificity of the kinase.

Acknowledgments

We wish to thank G. Tcherkez for excellent expertise for biochemical work and metabolomics, E. Bismuth and J.N. Pierre for help in some experiments and R. Boyer for excellent photographic work. The isolation of ppck1 mutants was financed by Génome (1997–2000) "Réalisation et exploitation d'une collection de mutants d'ADN-T chez *Arabidopsis thaliana*". We wish to thank the French Ministry of Education, Research and Technology (Ph.D. grants to P. Meimoun, A. Gousset-Dupont and B. Bouteiller), the CNRS and the Université Paris-Sud 11 for financial support.

References

[1] Chollet, R., Vidal, J. and O'Leary, M.H. (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 273–298.
[2] Nhiri, M., Bakrim, N., Pacquit, V., El Hachimi-Messouak, Z., Osuna, L. and Vidal, J. (1998) Ca^{2+}-dependent and -independent phosphoenolpyruvate carboxylase kinases in sorghum leaves: further evidence for the involvement of the calcium-independent protein kinase in the in situ regulatory phosphorylation of C_4 phosphoenolpyruvate carboxylase. Plant Cell Physiol. 39, 241–246.
[3] Terada, K., Kai, T., Okuno, S., Fujisawa, H. and Izui, K. (1990) Maize leaf phosphoenolpyruvate carboxylase: phosphorylation of Ser^{15} with a mammalian cyclic AMP-dependent protein kinase diminishes sensitivity to inhibition by L-malate. FEBS Lett. 259, 241–244.
[4] Nimmo, H.G. (2003) Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. Arch. Biochem. Biophys. 414, 189–196.
[5] Hartwell, J., Gill, A., Nimmo, G.A., Wilkins, M.B., Jenkins, G.I. and Nimmo, H.G. (1999) Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. Plant J. 20, 333–342.
[6] Taybi, T., Patil, S., Chollet, R. and Cushman, J.C. (2000) A minimal serine/threonine protein kinase circadianly regulates phosphoenolpyruvate carboxylase activity in crassulacean acid metabolism-induced leaves of the common ice plant. Plant Physiol. 123, 1471–1482.
[7] Tsuchida, Y., Furumoto, T., Izumida, A., Hata, S. and Izui, K. (2001) Phosphoenolpyruvate carboxylase kinase involved in C(4) photosynthesis in *Flaveria trinervia*: cDNA cloning and characterization. FEBS Lett. 507, 318–322.
[8] Fontaine, V., Hartwell, J., Jenkins, G.I. and Nimmo, H.G. (2002) *Arabidopsis thaliana* contains two phosphoenolpyruvate carboxylase kinase genes with different expression patterns. Plant Cell Environ. 25, 115–125.
[9] Fukayama, H., Tamai, T., Taniguchi, Y., Sullivan, S., Miyao, M. and Nimmo, H.G. (2006) Characterization and functional analysis of phosphoenolpyruvate carboxylase kinase genes in rice. Plant J. 47, 258–268.
[10] Shenton, M., Fontaine, V., Hartwell, J., Marsh, J.T., Jenkins, G.I. and Nimmo, H.G. (2006) Distinct patterns of control and expression amongst members of the PEP carboxylase kinase gene family in C_4 plants. Plant J. 48, 45–53.
[11] Furumoto, T., Izui, K., Quinn, V., Furbank, R.T. and von Caemmerer, S. (2007) Phosphorylation of phosphoenolpyruvate carboxylase is not essential for high photosynthetic rates in the C_4 species *Flaveria bidentis*. Plant Physiol. 144, 1936–1945.
[12] Gousset-Dupont, A., Lebouteiller, B., Monreal, J., Echevarria, C., Pierre, J.N., Hodges, M. and Vidal, J. (2005) Metabolite and post-translational control of phosphoenolpyruvate carboxylase from leaves and mesophyll cell protoplasts of *Arabidopsis thaliana*. Plant Sci. 169, 1096–1101.
[13] Meimoun, P., Ambard-Bretteville, F., Colas-des Francs-Small, C., Valot, B. and Vidal, J. (2007) Analysis of plant phosphoproteins. Anal. Biochem. 371, 238–246.
[14] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[15] Pacquit, V., Giglioli, N., Cretin, C., Pierre, J.N., Vidal, J. and Echevarria, C. (1995) Regulatory phosphorylation of C4 phosphoenolpyruvate carboxylase from Sorghum: an immunological study using specific anti-phosphorylation site-antibodies. Photosynth. Res. 43, 283–288.
[16] Noctor,G., Bergot, C., Mauve, C., Thominet, D., Lelarge-Trouverie, C. and Prioul, J.L. (2007) A comparative study of amino-acid analysis in leaf extracts by gas chromatography-time of flight-mass spectrometry and high performance liquid chromatography with fluorescence detection. Metabolomics, doi: 10.1007/s11306-007-0057-3.
[17] Novitskaya, L., Trevanion, S.J., Driscoll, S., Foyer, C.H. and Noctor, G. (2002) How does photorespiration modulate leaf aminoacid contents? A dual approach through modelling and metabolite analysis. Plant Cell Environ. 25, 821–835.